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# ***U.S. PATENT APPLICATION***

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***Invention:*** COMBINATION OF LACTIC ACID BACTERIA AND ITS USE FOR THE  
PREVENTION AND/OR TREATMENT OF INFECTIONS AND  
INFLAMMATORY CONDITIONS

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## ***SPECIFICATION***

Combination of lactic acid bacteria and its use for the prevention and/or treatment of infections and inflammatory conditions

5           The present invention relates to a combination of lactic acid bacteria and its use for making a food supplement, a hygiene product or a pharmaceutical preparation for the prevention and/or treatment of infections and inflammatory conditions  
10 caused by bacteria, viruses or fungi, especially in the mouth, vagina, urethra, nose, eyes and ears.

          Lactic acid bacteria are Gram-positive bacteria that produce lactic acid by the fermentation of glucose. *Streptococcus thermophilus* is also  
15 included in this definition by convention.

          It is well known that strains of lactic acid bacteria that produce  $H_2O_2$  can act as regulators of the bacterial flora in body orifices and on mucous membranes. It has been demonstrated that  $H_2O_2$ -producing  
20 lactic acid bacteria can antagonize *E. coli*, *N. gonorrhoea*, *G. vaginalis*, *C. trachomatis*, *U. urealyticum* and *B. bivius*. However, these bacteria are only of limited benefit when used in medical practice. This can be seen from the fact that  
25 preparations based on lactic acid bacteria (e.g. vaginal pessaries) intended for the treatment of infections by the above microorganisms (e.g. vaginitis) are not held in high regard by doctors, who prefer to

treat their patients with antibiotics or chemotherapeutic agents.

To the best of the inventor's knowledge, no antibacterial or flora-regulating action in body orifices and on mucous membranes has been attributed to arginine-utilizing lactic acid bacteria.

It has now been found surprisingly that the activity of  $H_2O_2$ -producing lactic acid bacteria is considerably potentiated by the addition of one or more strains of lactic acid bacteria that are capable of utilizing arginine. Arginine forms part of various small peptides found in biological fluids and it also occurs as free arginine. Many bacterial species utilize it for their own nutrition and growth. Arginine-utilizing lactic acid bacteria can therefore deprive other, pathogenic or potentially pathogenic bacteria of a certain quantity of arginine, which - though not enough to terminate their growth - makes them more susceptible to the action of the  $H_2O_2$  produced by the lactic acid bacteria.

The present invention therefore provides a combination of lactic acid bacteria comprising:

- (a) a first component consisting of at least one strain of  $H_2O_2$ -producing lactic acid bacteria, and
- (b) a second component consisting of at least one strain of arginine-utilizing lactic acid bacteria.

The strain of lactic acid bacteria in component (a) is preferably chosen from a group made up of the strains of the species *Lactobacillus crispatus*,

*Lactobacillus salivarius* and *Lactobacillus casei*, while the strain of lactic acid bacteria in component (b) is chosen from a group made up of the strains of the species *Lactobacillus brevis*, *Lactobacillus gasseri* and  
5 *Lactobacillus fermentum*. More especially, the strain of lactic acid bacteria in component (b) is the *Lactobacillus brevis* CD2 strain deposited in the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, on February 6, 1998 with  
10 access number DSM 11988 under the Budapest Treaty, or mutants or derivatives thereof.

The ratio of the number of bacteria in component (a) to the number of bacteria in component (b) is preferably from 1 : 100 to 100 : 1, and more  
15 especially from 1 : 5 to 5 : 1, the most preferred ratio being 1 : 1.

The combination can be administered in the unit dosage form comprising from  $1 \times 10^2$  to  $5 \times 10^{11}$  bacteria of component (a) and from  $1 \times 10^2$   
20 to  $5 \times 10^{11}$  bacteria of component (b), preferably  $1 \times 10^9$  bacteria of component (a) and  $3 \times 10^9$  bacteria of component (b).

The combination can also be administered in the form of tablets, sucking tablets, sweets, chewing  
25 gum, gelatin capsules, pessaries, suppositories and micro-enemas, as well as pellets, dental creams and gels, denture powders, mouthwashes, dentifrices, sprays, suspensions and ointments.

According to another embodiment of the invention, the combination additionally comprises at least one other strain of lactic acid bacteria chosen from a group made up of:

- 5 *Lactobacillus acidophilus*, *Lactobacillus buchneri*,  
*Lactobacillus casei*, *Lactobacillus cateniforme*,  
*Lactobacillus cellobiosus*, *Lactobacillus crispatus*,  
*Lactobacillus curvatus*, *Lactobacillus delbrueckii*,  
*Lactobacillus jensenii*, *Lactobacillus leichmannii*,  
10 *Lactobacillus minutus*, *Lactobacillus plantarum*,  
*Lactobacillus salivarius*, *Lactobacillus brevis*,  
*Lactobacillus gasseri*, *Lactobacillus fermentum*,  
*Bifidobacterium adolescentis*, *Bifidobacterium*  
*angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium*  
15 *breve*, *Bifidobacterium catenulatum*, *Bifidobacterium*  
*dentium*, *Bifidobacterium eriksonii*, *Bifidobacterium*  
*infantis*, *Bifidobacterium longum*, *Bifidobacterium*  
*plantarum* and *Streptococcus thermophilus*.

The combination can also comprise vitamins,  
20 quaternary ammonium bases, mineral salts, antioxidants  
and anti-plaque agents.

The invention also relates to the use of a combination of lactic acid bacteria comprising:

(a) a first component consisting of at least one  
25 strain of H<sub>2</sub>O<sub>2</sub>-producing lactic acid bacteria and

(b) a second component consisting of at least one strain of arginine-utilizing lactic acid bacteria, for making a food supplement, a hygiene product or a pharmaceutical preparation for the prevention and/or

treatment of infections and inflammatory conditions caused by bacteria, viruses or fungi, especially in the mouth, vagina, urethra, nose, eyes and ears. These infections and inflammatory conditions include

5 gingivitis, periodontitis, mucositis and stomatitis caused by drugs and/or physical agents, Behçet's syndrome, diakeratosis of the oral cavity, glossitis, sore throat, sialadenitis, sialolithiasis, pemphigus, *Lichen planus*, Sjögren's syndrome, vaginosis,  
10 vaginitis, urethritis, prostatitis, proctitis, otitis, conjunctivitis, rhinitis, sinusitis, leucoplakia, aphthae, herpes, and infections with *Helicobacter pilori* in the oral cavity.

The combination can also be used to advantage  
15 for the treatment of the oral cavity as an oral deodorant, antiinflammatory, anti-carries and/or anti-plaque agent.

The following examples serve to illustrate the various aspects of the invention in more detail but  
20 should not be construed as in any way limiting the invention.

#### Example 1

The inhibitory effect of: H<sub>2</sub>O<sub>2</sub>-producing lactic acid bacteria (Component A); arginine-utilizing  
25 lactic acid bacteria (Component B); and the combination of the two strains, specifically in the ratio 1 : 1 (Combination AB); on the growth of potentially pathogenic bacteria was evaluated.

20                   The characterization of the bacteria as  $H_2O_2$   
producers was done by a classical benzidine peroxidase  
reaction, which reveals  $H_2O_2$ -producing colonies of  
bacteria by a blue coloration. The activity of  
arginine dehydrolase was determined to evaluate the  
25   ability of the lactic acid bacteria to utilize arginine  
(M.C. Manca de Nadra, *Milchwissenschaft*, **37** (1982)  
pp. 669-670].

The lactic acid bacteria were obtained from the American Type Culture Collection (ATCC), Rockville, USA.

<u>Bacterial strain</u>	<u>Halo of inhibition</u> <u>(anti-G. vaginalis</u> <u>activity, mm)</u>
H <sub>2</sub> O <sub>2</sub> producer (Component A)	
<i>Lactobacillus crispatus</i> (ATCC 39197)	75
<i>Lactobacillus salivarius</i> (ATCC 11741)	60
<i>Lactobacillus crispatus</i> + <i>Lactobacillus salivarius</i>	63
Arginine utilizer (Component B)	
<i>Lactobacillus brevis</i> (ATCC 14869)	0
<i>Lactobacillus fermentum</i> (ATCC 14931)	2
<i>Lactobacillus brevis</i> + <i>Lactobacillus</i> <i>fermentum</i>	0
Combination AB (ratio of A to B 1 : 1)	
<i>Lactobacillus crispatus</i> + <i>Lactobacillus brevis</i>	112
<i>Lactobacillus crispatus</i> + <i>Lactobacillus fermentum</i>	100



<i>Lactobacillus salivarius</i> +	
<i>Lactobacillus brevis</i>	117
<i>Lactobacillus salivarius</i> +	
<i>Lactobacillus fermentum</i>	104

Bacterial strain	Halo of inhibition (anti- <i>S. mutans</i> activity, mm)
H <sub>2</sub> O <sub>2</sub> producer (Component A)	
<i>Lactobacillus crispatus</i> (ATCC 39197)	98
<i>Lactobacillus salivarius</i> (ATCC 11741)	102
<i>Lactobacillus crispatus</i> +	
<i>Lactobacillus salivarius</i>	99
Arginine utilizer (Component B)	
<i>Lactobacillus brevis</i> (ATCC 14869)	0
<i>Lactobacillus fermentum</i> (ATCC 14931)	1
<i>Lactobacillus brevis</i> + <i>Lactobacillus</i>	
<i>fermentum</i>	1

Combination AB (ratio of A to B 1 : 1)

<i>Lactobacillus crispatus</i> +	
<i>Lactobacillus brevis</i>	118
<i>Lactobacillus crispatus</i> +	
<i>Lactobacillus fermentum</i>	126
<i>Lactobacillus salivarius</i> +	
<i>Lactobacillus brevis</i>	121
<i>Lactobacillus salivarius</i> +	
<i>Lactobacillus fermentum</i>	120

Example 2

Sucking tablets with the following unit composition were prepared:

5

Combination AB	4000 million
( <i>Lactobacillus salivarius</i> +	
<i>Lactobacillus brevis</i> , ratio 1 : 1)	
Mannitol	400 mg
Saccharin	5 mg
Polyoxyethylene	50 mg
Mg stearate	15 mg
Talc	25 mg
Silica	5 mg

These tablets were administered to four volunteers who were told not to clean their teeth or use chewing gum during the previous week. The subjects

10 took three tablets a day for one week, after meals,

allowing the tablets to dissolve in their mouth.

Clinical evaluations were performed for both the dental plaque index and the gingival plaque index.

5 For the dental plaque the following scoring system was used on six teeth (first upper molar on the right; upper central incisor on the left; first upper premolar on the left; first lower molar on the left; lower central incisor on the right; first lower premolar on the right):

10

- 0 - no plaque at all
- 1 - no visible plaque
- 2 - visible plaque
- 3 - very obvious plaque.

15

The following scoring system was adopted for evaluating the gingival plaque, using the margin of the six teeth mentioned above:

- 0 - no inflammation
- 20 1 - slight inflammation
- 2 - moderate inflammation, with bleeding on contact
- 3 - marked inflammation, with a tendency to spontaneous bleeding.

25

The combined data obtained for the six teeth of the four volunteers were as follows:

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<u>Subject</u>	<u>Dental plaque index</u>		<u>Gingival plaque index</u>	
	0	7 days	0	7 days
No. 1	8	2	7	1
No. 2	9	3	10	3
No. 3	15	5	6	2
No. 4	15	7	8	3

### Example 3

5                   Four subjects with a clinical and histological diagnosis of recurrent aphthous ulcers were treated for ten days with six sucking tablets a day, whose composition is given in Example 2.

10                   All the patients treated showed a complete cure of the ulcers at the end of the ten-day course, and none of them had new ulcers during the following month.

15                   To improve the flavour and appearance of the bacterial combination AB, suitable colouring agents and sweeteners such as saccharin, mint oil and xylitol can be added, as is customary and well known to those skilled in the art.

20                   The combination AB can be administered in the form of pellets, sweets, chewing gum, gelatin capsules, dental creams and gels, denture powders, mouthwashes, dentifrices, tablets, pessaries, suppositories, sprays, suspensions and micro-enemas.

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Example 4Preparation of a toothpaste

Toothpaste base

## 5 Percentage composition:

Calcium phosphate dihydrate	37.5%
Glycerol (85% in water)	30.0%
Flavour (peppermint oil)	1.0%
Sodium carboxymethylcellulose	1.0%
10 Purified water	20.8%
Sodium saccharin (1% aq. solution)	2.5%
Sodium lauryl sulphate	2.0%
Purified water	<u>5.2%</u>
	100.0%

## 15 Composition by weight:

Calcium phosphate dihydrate	337.5 g
Glycerol (85% in water)	270.0 g
Flavour (peppermint oil)	9.0 g
Sodium carboxymethylcellulose	9.0 g
20 Purified water	187.2 g
Sodium saccharin (1% aq. solution)	22.5 g
Sodium lauryl sulphate	18.0 g
Purified water	<u>48,8 g</u>
	900.0 g

25

The glycerol was added to the calcium phosphate dihydrate, which had been ground and passed through a 50-mesh screen. The mixture was allowed to undergo hydration, giving a dense homogeneous paste.

The flavour (1% of peppermint oil) was added at this point.

The sodium carboxymethylcellulose was hydrated in water overnight (concentration of 4.6% in 5 purified water).

The solution of sodium saccharin was added to the resulting thick gel.

The polymer gel was poured into a mortar already containing the calcium phosphate that had been 10 hydrated with glycerol, and the components were vigorously mixed.

An approximately 28% solution of sodium lauryl sulphate in purified water was prepared separately.

15 The sodium lauryl sulphate solution was then added to the thick dicalcium phosphate paste.

The resulting thick homogeneous paste, which had good rheological properties, was mixed for a few minutes and then refined by passing it through a 20 refiner with rollers. This gave a snow-white homogeneous paste with a pleasant mint aroma.

38.5 g of lyophilized lactic acid bacteria (*L. salivarius* + *L. brevis*, 1 : 1;  $10^{10}$  CFU/g) were passed through a 50-mesh screen and added in small 25 portions to the rest (770 g) of the above toothpaste base. The resulting homogeneous paste had a slightly pale brown colour and a mint odour.

Example 5Preparation of fast-release vaginal tablets

Vaginal tablets coated with an effervescent layer were prepared by wet granulation. The tablets  
5 weighed 2100 mg each and contained 100 mg of the combination of lactic acid bacteria as the active substance.

Each vaginal tablet had the following composition:

10	Lyophilized and screened lactic acid bacteria ( $30 \times 10^9$ of <i>L. brevis</i> , $30 \times 10^9$ of <i>L. salivarius</i> and $90 \times 10^9$ of <i>L. plantarum</i> per gram)	100.0 mg
	Lactose	1368.0 mg
15	Cornstarch	246.0 mg
	Adipic acid	192.0 mg
	Sodium bicarbonate	150.0 mg
	Magnesium stearate	30.0 mg
	Stearic acid	9.0 mg
20	Colloidal silica	<u>5.0 mg</u>
		2100.0 mg

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